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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12P 19/34, C12Q 1/68
C12N 9/12, C07H 15/12, 17/00

A1

(11) International Publication Number: WO 91/16446

(43) International Publication Date: 31 October 1991 (31.10.91)

(21) International Application Number: PCT/US90/01631

(22) International Filing Date: 13 April 1990 (13.04.90)

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(81) Designated States: AT (European patent), AU, BB, BE (European patent), BG, BR, CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, MW, NL (European patent), NO, RO, SD, SE (European patent), SU.

Published
With international search report.

(54) Title: *IN VITRO* DNA SYNTHESIS REACTIONS USING PHI 29 DNA POLYMERASE AND A DNA FRAGMENT ENCODING SAID POLYMERASE

(57) Abstract

An improved method for determining the nucleotide base sequence of a DNA molecule. The method includes annealing the DNA molecule with a primer molecule able to hybridize to the DNA molecule; incubating the annealed mixture in a vessel containing four different deoxynucleoside triphosphates, a DNA polymerase, and one or more DNA synthesis terminating agents which terminate DNA synthesis at a specific nucleotide base, wherein each of the agent terminates DNA synthesis at a different nucleotide base; and separating the DNA products of the incubating reaction according to size, whereby at least a part of the nucleotide base sequence of the DNA can be determined. The improvement is provision of a DNA polymerase which is a Φ29-type DNA polymerase.

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IN VITRO DNA SYNTHESIS REACTIONS USING PHI29 DNA POLYMERASE AND A DNA FRAGMENT ENCODING SAID POLYMERASE

Background of the Invention

This invention relates to DNA polymerases suitable for DNA sequencing.

DNA sequencing involves the generation of four populations of single-stranded DNA fragments, having one defined terminus and one variable terminus. The variable terminus always terminates at a specific given nucleotide base (either guanine (G), adenine (A), thymine (T), or cytosine (C)). The four different sets of fragments are each separated on the basis of their length, on a high resolution polyacrylamide gel; each band on the gel corresponds colinearly to a specific nucleotide in the DNA sequence, thus identifying the positions in the sequence of the given nucleotide base.

Generally there are two methods of DNA sequencing. One method (Maxam and Gilbert sequencing) involves the chemical degradation of isolated DNA fragments, each labeled with a single radiolabel at its defined terminus, each reaction yielding a limited cleavage specifically at one or more of the four bases (G, A, T or C). The other method (dideoxy sequencing) involves the enzymatic synthesis of a DNA strand. Four separate syntheses are run, each reaction being caused to terminate at a specific base (G, A, T or C) via incorporation of the appropriate chain terminating dideoxynucleotide. The latter method is preferred since the DNA fragments are uniformly labelled (instead of end labelled) and thus the larger DNA fragments contain increasingly more radioactivity. Further, ^{35}S-labelled nucleotides can be used in place of ^{32}P-labelled nucleotides, resulting in sharper

definition; and the reaction products are simple to interpret since each lane corresponds only to either G, A, T or C. The nzymes used for most dideoxy sequencing is the Escherichia coli DNA-polymerase I large fragment ("Klenow"), AMV reverse transcriptase, and T7 DNA polymerase (Tabor et al., U.S. Patent 4,795,699). The T7 DNA polymerase used for sequencing is said to be advantageous over other DNA polymerases because it is processive, has no associated exonuclease activity, does not discriminate against nucleotide analog incorporation, and can utilize small oligonucleotides as primers. These properties are said to make the polymerase ideal for DNA sequencing. Id.

Summary of the Invention

In a first aspect, the invention features an improved method for determining the nucleotide base sequence of a DNA molecule. The method includes annealing the DNA molecule with a primer molecule able to hybridize to the DNA molecule; incubating the annealed mixture in a vessel containing four different deoxynucleoside triphosphates, a DNA polymerase, and one or more DNA synthesis terminating agents which terminate DNA synthesis at a specific nucleotide base, wherein each the agent terminates DNA synthesis at a different nucleotide base; and separating the DNA products of the incubating reaction according to size, whereby at least a part of the nucleotide base sequence of the DNA can be determined. The improvement is provision of a DNA polymerase which is a ϕ29-type DNA polymerase.

By ϕ29-type DNA polymerase is meant any DNA polymerase isolated from the related phages which contain a terminal protein used in the initiation of replication of DNA. These phages are generally described by Salas, 1 The Bacteriophages 169, 1988.

These phages ar closely related in the structure of th ir DNA polymerases, some differing by as few as 6 amino acid changes with 5 of those amino acids being replaced by similar amino acids. These phages have a short inverted terminal repeat sequence of length between about 6 and 300 nucleotides. These polymerases have a highly active 3'-5' exonuclease activity, but no 5'-3' exonuclease activity. Surprisingly, although they are related to the T4 family of DNA polymerases, they are able to adequately recognize chain terminating agents such as dideoxynucleosides and therefore are useful for DNA sequencing. This ability is even more surprising since the exonuclease is known to recognize both deoxy and dideoxy ADP. Blanco et al. 13 Nuc. Acid. Res. 1239, 1246, 1985.

In preferred embodiments, the ϕ29-type DNA polymerase is that polymerase in cells infected with a ϕ29-type phage; the ϕ29-type DNA polymerase is chosen from ϕ29, Cp-1, PRD1, ϕ15, ϕ21, PZE, PZA, Nf, M2Y, B103, SF5, GA-1, Cp-5, Cp-7, PR4, PR5, PR722, and L17; the ϕ29-type DNA polymerase is a modified polymerase, having less than ten percent of the exonuclease activity of the naturally-occurring polymerase, most preferably the polymerase has less than one percent, and even more preferably has substantially no exonuclease activity; and the terminating agent is a dideoxynucleotide.

In a related aspect, the invention features a kit for DNA sequencing including a supply of ϕ29-type DNA polymerase, together with a supply of a chain terminating agent. By kit is meant a container designed to keep these two components separated from each other, preferably in condition for use in a DNA sequencing reaction.

In another related aspect, th invention featur s a DNA fragm nt encoding a modified ϕ29-type DNA polymerase, wherein the polymerase has sufficient DNA polymerase activity for use in DNA sequencing, and an exonuclease activity which is less than 10% the activity of the corresponding naturally occurring ϕ29-type DNA polymerase.

By corresponding is meant that the modified polymerase is derived from a naturally occurring polymerase, generally by in vitro mutagenesis of the DNA sequence enccding the latter polymerase, and the latter is the corresponding polymerase.

In preferred embodiments, the DNA fragment is modified to substantially eliminate the naturally-occurring exonuclease activity; and the DNA fragment includes a DNA sequence encoding a ϕ29 DNA polymerase in which the amino acid moiety at position 12, 14, or 16 of the polymerase is replaced by an alanine moiety.

The invention also features a ϕ29-type DNA polymerase produced from the above described DNA fragments.

In another aspect, the invention features an improved method for amplification of a DNA sequence. The method includes annealing a first and second primer to opposite strands of a double-stranded DNA sequence, and incubating the annealed mixture with a DNA polymerase. The improvement includes employing as the DNA polymerase a ϕ29-type DNA polymerase.

In preferred embodiments, the first and second primers have their 3' ends directed towards each other after annealing; the method further includes, after the incubation step, denaturing the resulting DNA, annealing the first and second primers to the denatured DNA and

incubating the last annealed mixture with the polymerase; the cycle of denaturing, annealing, and incubating is r p ated from 10-40 times; the ϕ29-type DNA polymerase is selected from: ϕ29, Cp-1, PRD1, ϕ15, ϕ21, PZE, PZA, Nf, M2Y, B103, SF5, GA-1, Cp-5, Cp-7, PR4, PR5, PR722, and L17; the DNA polymerase exhibits less than 10% of the naturally-occurring exonuclease activity exhibited by the corresponding naturally-occurring polymerase, most preferably the polymerase has no detectable exonuclease activity.

In a further aspect, the invention features a method for production of DNA molecules of greater than 10 kilobases in length. The method includes providing a template DNA molecule; annealing a primer with the template molecule; and incubating the annealed primer and template molecules in the presence of a ϕ29-type DNA polymerase, and a mixture of four different deoxynucleoside triphosphates.

The invention also features a method for amplification of a heterologous DNA molecule including covalently bonding a ϕ29-type terminal DNA sequence at one end of the DNA molecule to form a product; and incubating the product in the presence of a ϕ29-type DNA polymerase and a terminal protein (see below) of a ϕ29-type DNA polymerase.

By heterologous is meant any DNA which does not naturally occur within a ϕ29-type phage DNA molecule. This includes DNA encoding any desired protein.

A terminal DNA sequence is a sequence which naturally occurs at one or both ends of a ϕ29-type phage DNA which may be between 6 and 300 bases long. This sequence is specifically recognized and bound by a terminal protein, for example, the p3 protein of ϕ29.

In preferred embodiments, the method includes

providing a φ29-type terminal DNA sequence at each end of the DNA molecule to be amplified; the terminal sequence is provided on a DNA fragment of less than 500 nucleotides; and the terminal protein is the terminal protein of the φ29-type phage in which the φ29-type DNA polymerase naturally occurs.

This invention provides a DNA polymerase which is highly processive, and may be produced with a low exonuclease activity. The high processivity of the polymerase makes it suitable, not only for DNA sequencing, but also for amplification of very large fragments of DNA (in excess of 10 kilobases in length). This makes the polymerase useful in a polymerase chain reaction (PCR)-type procedure or in replicative-type, protein primed, extension reactions. These long lengths of DNA are of use in forensic work, when small samples of DNA are available, and for restriction fragment length polymorphism analysis.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawing will first briefly be described.

Drawing

The Figure is a represention of the amino acid sequence of various DNA polymerases showing sites of homology between the polymerases. ExoI, ExoII and ExoIII refer to the three regions of amino acid homology found among the different DNA polymerases compared in the Figure. Stars indicate the E. coli DNA polymerase I residues involved in either metal binding, or exonucleolytic catalysis. Asterisks indicate the E. coli DNA polymerase I residues involved in single-stranded DNA binding. Boxes shown by lines or

arrows, and lett red or numb red, are α-helix and ß-sheet regions r spectively of DNA polymeras I.

DNA Polymerase

In general, a DNA polymerase of this invention is processive and has naturally-occurring exonuclease activity associated with it. In some preferred embodiments, the DNA polymerase has little or no associated exonuclease activity. These polymerases also have a strand-displacement activity.

By processive is meant that the DNA polymerase is able to continuously incorporate nucleotides using the same primer template, without dissociating from either or both the primer or the template molecules, under conditions normally used for DNA sequencing extension reactions, or other primer extension reactions. Generally, polymerases of the present invention will remain bound to the extended primer or template for at least 1-2 kilobases, generally at least 5kb-10kb, under suitable environmental conditions.

The ability of the polymerases of this invention to produce strand-displacement is advantageous in this invention beause, in combination with high processivity, it allows synthesis of long DNA molecules of at least 70kb, or even greater. Strand displacement activity is measured by any standard technique, for example, a polymerase may be incubated in a mixture with a single-stranded circular DNA molecule (e.g., M13) and a primer. If DNA molecules of length greater than the original circular molecule are synthesized, then the polymerase is able to displace DNA strands of a double-stranded molecule and continue to synthesize DNA--thus, it has a strand displacement activity. Such activity is generally present in a single protein molecule, e.g., p2 of ϕ29, and does not require energy

in the form of ATP or its equivalent, utilizing only the standard deoxynucleoside triphosphates required to synthesize DNA. This activity is also observed when DNA synthesis is initiated by a terminal protein, e.g., p3 of ϕ29.

The exonuclease activity associated with DNA polymerases of this invention does not appear to significantly interfere with the use of the polymerase in a DNA sequencing reaction. However, it is preferred that the level of exonuclease activity be reduced to a level which is less than 10% or 1%, preferably less than 0.1% of the activity normally associated with DNA polymerases isolated from cells infected with naturally-occurring bacteriophage.

The DNA polymerases of this invention include polymerases which have been genetically modified to reduce the exonuclease activity of that polymerase, as well as those which are substantially identical to a naturally-occurring ϕ29-type DNA polymerase or a modified polymerase thereof, or to the equivalent enzymes enumerated above. Each of these enzymes can be modified to have properties similar to those of the ϕ29 DNA polymerase. It is possible to isolate the enzyme from phage-infected cells directly, but preferably the enzyme is isolated from cells which over-produce it.

By substantially identical is meant that the enzyme may contain amino acid substitutions which do not affect the overall properties of the enzyme. One example of a particularly desirable amino acid substitution is one in which the natural enzyme is modified to remove any exonuclease activity. This modification may be performed by genetic or chemical means.

As an example of this invention we shall describe the use of ϕ29 DNA polymeras in a variety of useful procedures. This example is not meant to be limiting to the invention; those skilled in the art will recognize that any of the above enumerated DNA polymerases can be similarly used in the manner described below.

ϕ29 DNA Polymerase

Bacteriophage ϕ29 is a linear double-stranded DNA molecule having a protein of 31 kD covalently linked at the 5' end. This terminal protein, termed p3, is the product of viral gene 3, and is linked to the DNA by a phosphoester-bond between the OH group of a serine residue and 5'-dAMP. ϕ29 replication is initiated at either DNA end by a protein priming mechanism in which a free molecule of the terminal protein p3 reacts with dATP to form a protein-p3-dAMP covalent complex that provides the 3' OH group needed for elongation. The initiation reaction requires, in addition to the gene 3 product and the ϕ29 DNA-protein p3 template, the product of the viral gene 2 (p2), which is the DNA polymerase. Protein p2 produced from gene 2 has a molecular weight of 66.5 kD. Associated with protein p2 is a 3'-5' exonuclease activity active on single stranded and to some extent on double stranded DNA. Protein p2 may be purified by standard procedure from E. coli cells harboring a gene 2 containing recombinant plasmid, as described by Blanco et al., 29 Gene 33, 1984. The protein may be further purified by passage over a phosphocellulose column, as described by Blanco et al., 13 Nuc. Acid. Res. 1239, 1985. Blanco et al., id., also describe an exonuclease assay suitable for determination of inactivation of the exonuclease activity by genetic manipulation, as described below.

Other enzym s associated with p2 and p3 in bacteriophage ϕ29 include p5 and p6, which increase th efficiency of polym rization by p2, as described by Salas, 109 Current Topics in Microbiology and Immunology 89, 1983.

Exonuclease Mutants

We shall now briefly describe the cloning of ϕ29 DNA polymerase and the manipulation of the p2 gene to produce examples of exonuclease mutants useful in this invention.

The starting plasmid was pBw2, which is a pBR322 derivative containing gene 2 of phage ϕ29, coding for the ϕ29 DNA polymerase, and including its ribosome-binding sequence (RBS) (Blanco et al. 29 Gene 33, 1984). In this construction the putative ATG initiation codon for the ϕ29 DNA polymerase is located 30 bp downstream a unique HindIII restriction site. Plasmid pBw2 was linearized with Hind III and subjected to a controlled digestion with the nuclease Bal31. The DNA was then digested with the restriction nuclease ScaI, which cuts 444 base pairs downstream gene 2, and the 5' protruding ends were filled-in with the Klenow fragment of E. coli DNA polymerase I. The DNA fragment containing gene 2 was ligated with the T4 DNA ligase to plasmid pAZe3ss (Zaballos et al., 58 Gene 67, 1987) and digested with NcoI, whose 5' protruding ends were then filled-in using Klenow fragment. The ligation product was used to transform competent E. coli M72 cells (lysogenic for bacteriophage λ and containing the temperature-sensitive cI857 repressor) and ampicillin-resistant bacteria selected. The latter were replica-plated in plates containing ampicillin (100 μ/ml) by growing them overnight at 30°C, followed by 3 h at 42°C. The colonies were transfered to nitrocellulose filters and lysed with 0.1% sodium

dodecyl sulfate. Th filters were wash d, incubated with rabbit anti-ϕ29 DNA polymerase serum (produced by standard procedure) and the ϕ29 DNA polymerase-containing colonies were detected by incubation with [^{125}I] protein A followed by autoradiography. DNA sequencing of the selected clones allowed selection of the recombinant plasmids pAZw200 and pAZa203, which include ϕ29 DNA starting at the ATG triplets corresponding to position 2869-2867 and 2860-2858, respectively, in the open reading frame ccding for p2, from the left ϕ29 DNA end (Yoshikawa et al., 17 Gene, 323, 1982). When the E. coli M72 cells, transformed with the recombinant plasmids pAZw200 or pAZa203, containing the gene coding for the ϕ29 DNA polymerase under the control of the P_L promoter of bacteriophage λ and with the RBS of gene ner of bacteriophage Mu, were grown at 30°C and then shifted to 42°C for 20 min to inactivate the λ CI857 repressor, followed by 2 h at 38°C, enzymatically active ϕ29 DNA polymerase was synthesized. About 150 and 300 µg of highly purified ϕ29 DNA polymerase was obtained per g of cells transformed with the recombinant plasmids pAZw200 and pAZa203, respectively.

The EcoRI-Hind III fragment from the recombinant plasmid pAZw200, containing the ϕ29 DNA polymerase gene and the RBS of gene ner of bacteriophage Mu was ligated, using T4 DNA ligase, to the EcoRI-HindIII sites of the replicative form of bacteriophage M13mp19. E. coli JM103 cells were transfected with such DNA and white plaques were selected in plates containing X-gal and isopropilthiogalactoside (IPTG). The selected plaques were amplified in liquid medium and the replicative form was isolated to check (by restriction analysis) the

presence of the desired EcoRI-HindIII fragment. The single-stranded DNA was also isolated and used for site-directed mutagenesis, carried out as described by Nakamaya et al., 14 Nucl. Acids Res. 9679, 1986. The synthetic oligodeoxynucleotides used for the site-directed mutagenesis were:

1) 5' AGTTGTGCCTTTGAGAC
2) 5' GACTTTGCGACAACTAC
3) 5' CTCAAATTTGCCGGAGC

The recombinant clones containing point mutations were selected by hybridization to the corresponding mutagenic oligonucleotides 5' [^{32}P]-labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP. Single-stranded DNA was isolated from the selected clones and the sequence of the complete DNA polymerase gene was determined to check that each clone contained only the desired mutation. The EcoRI-BstBI fragment from the different clones was ligated with T4 DNA ligase to the same sites of plasmid pABw2, which contains the EcoRI-HindIII fragment of plasmid pAZw200 cloned at the corresponding sites of plasmid pT7-3 of the pT7 series (Tabor et al. 82 Proc. Natl. Acad. Sci. USA, 1074, 1985), under the control of the ϕ10 promoter of bacteriophage T7. This EcoRI-BstBI fragment replaces the wild-type sequence in that region by the corresponding mutant sequence. In this way, the recombinant plasmids pABn2D12A, pABn2E14A, pABn2D66A, pABn2D12AD66A and pABn2E14AD66A were selected, containing the corresponding amino acid changes from the amino-terminal end of the ϕ29 DNA polymerase. The recombinant plasmids were used to transform E. coli BL21 (DE3) cells containing the bacteriophage T7 RNA polymerase gene in the host DNA under the control of the

lac uv5 promoter (Studier et al., 189 J. Mol. Biol. 113, 1986) being, therefore, inducible by IPTG. The ampicillin-resistant bacteria were analyzed for the presence of recombinant plasmids. Expression of the ϕ29 DNA polymerase mutant proteins was obtained by addition of 1 mM IPTG to E. coli cells containing the recombinant plasmids, grown at 37°C and incubated for 1 h at 37°C. Five different mutant proteins were obtained, with the following amino acid changes: 1) alanine at position 12 (with reference to the first methionine in the gene encoding p2) in place of the natural aspartic acid (D12A); 2) alanine at position 14 instead of glutamic acid (E14A); 3) alanine at position 66 instead of aspartic acid (D66A); 4) alanine at positions 12 and 66 instead of aspartic acid (D12A, D66A); and 5) alanine at position 14 and 66 (E14A, D66A). The different mutant proteins were purified and their 3'-5' exonuclease activity determined by the above standard assay to be 100-1000 fold lower than that of the wild-type naturally occurring ϕ29 DNA polymerase.

Deposits

Strains pAZW200 (wild type p2 gene), pKC30A1 (wild type p3 gene), pABN2D12AD66A (exonuclease deficient p2 gene having alanine at positions 12 and 66) have been deposited on March 24, 1989, with the ATCC and assigned numbers 67920, 67918, 67919, respectively.

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Referring to the Figure, the oligonucleotides used to form the above mutants were selected by taking into account the amino acid sequence homology with other polymerases and those mutations known to reduce exonuclease activity of DNA polymerase I. Derbyshire et al. 240 Science 199, 1988. Other mutations which are likely to produce suitable exonuclease mutants are shown in the black boxes. Generally, the amino acid at these portions is either deleted or replaced with a different amino acid. Large deletions or multiple replacement of amino acids are also useful in this invention. After mutagenesis, the level of exonuclease activity is measured and the amount of DNA polymerase activity determined to ensure it is sufficient for use in this invention (e.g., for DNA sequencing), being processive and having strand displacement activity.

Uses

DNA polymerases of this invention are useful in the following methods:

Filling in the 3' recessed termini created by digestion of DNA with restriction enzymes; labelling the termini of DNA fragments with protruding 5' ends (filling in reaction); labelling the termini of blunt-ended DNA fragments or DNA fragments with protruding 3' termini (exchange reaction); removing the 3' protruding termini of DNA fragments; labelling DNA fragments for use as hybridization probes by partial digestion of double-stranded DNA using the naturally associated 3'-5' exonuclease activity, followed by a

filling reaction with labelled dNTPs (displac ment reaction); synth sis of long (over 5-10kb) as well as short single-stranded DNA probes containing multiple copies of a desired sequence, obtained by strand-displacement synthesis on single stranded DNA, such long probes may be labelled with labelled dNTPs at a high specific activity; random labelling of double-stranded DNA at a high specific activity by using degenerated oligonucleotide primers; second-strand cDNA synthesis in cDNA cloning; sequencing DNA using a Sanger-type dideoxy system (Sanger et al. 74 Proc. Natl. Acad. Sci. USA 5463, 1977) on single- and double-stranded DNA templates; sequencing DNA by a plus/minus-type method (Sanger et al., 94 J. Mol. Biol. 441, 1975); random mutagenesis of single- and double-stranded DNA templates by using an exonuclease-deficient DNA polymerase under conditions of low DNA replication fidelity; site-directed mutagenesis on double stranded DNA templates; gene amplification or synthesis of long double-stranded DNA fragments using synthetic oligonucleotides as primers; and amplification or synthesis of double-stranded DNA fragments using a ϕ29-type DNA replication system including a ϕ29-type DNA polymerase, a terminal protein, any accessory proteins necessary to enhance the reaction, and a ϕ29-type DNA-protein p3 template.

The ϕ29-type DNA polymerases are particularly useful for DNA sequencing, performing a polymerase chain reaction, and for amplification without the need for temperature cycling to produce extremely long strands of DNA. These methods will now be discussed in detail.

Example 1: P.C.R.

There follows an example of a polymerase chain reaction using ϕ29 DNA polymerase. In general, the

DNA polym ras may simply be used in place of Klenow or Taq polymerases.

0.1 pmol of target DNA are mixed with 300 pmol each of selected oligonucleotides (15-20 mers), and 75 nmol of each deoxynucleoside triphosphate (1N5 mM) in 50 µl of a buffer containing 50 mM Tris-HCl (pH 7.5) and 10 mM magnesium chloride. The solution is brought to 95°C for 10 minutes, and cooled to 30°C for 1 min in a waterbath. 1 µl containing 20 ng of ϕ29 DNA polymerase (either wild type or an exonuclease mutant) is added to the mixture and the reaction allowed to proceed for 5 min at 30°C, after which the cycle of heating, cooling, adding enzyme, and reacting is repeated about nine times. The polymerase used is purified by standard procedures.

Prior polymerases used in polymerase chain reactions failed to provide DNA fragments in the size range greater than about 2 kilobases (Saiki et al., 239 Science 487, 1988; Keohavong et al., 71 Gene 211, 1988). This relative short size is probably due to the secondary structure and hinderance produced by reannealing of the DNA fragment, which impedes the progress of these DNA polymerases. Because ϕ29 DNA polymerase has a high processivity and strand displacement ability, it is an ideal enzyme for DNA amplification to produce long amplified molecules.

Example 2: DNA Sequencing

For DNA sequencing, the sequence procedure using single-stranded DNA as a template was essentially as described by Tabor et al., 84 Proc. Natl. Acad. Sci. USA 4767, 1987 with some modification.

In the annealing reaction, the annealing reaction mixture (20 µl) contained 2.5 µg of template DNA, 60 ng of primer (a 10-fold molar ratio to

the template) in a buffer containing 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 75 mM NaCl. The mixture was heated to 65°C for 15 min and then cooled to room temperature (20-25°C) over 30 min.

In the labelling reaction, a single labelling reaction was used for all four termination reactions. To the annealing mixture (20 µl) was added 20 µl of a mixture containing 0.6 µM each dGTP, dTTP, dCTP and [α-^{32}P]dATP, 2 mM dithiothreitol, 100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$ and 8% glycerol. Labelling was started by addition of the Φ29 DNA polymerase (either wild type or exonuclease deficient, 150 ng). Incubation was at room temperature for 5 min, at which time the reaction was complete. Four aliquots (8 µl each) of the "labelling" reaction mixture were used for the "termination" reactions.

In the extension-termination reaction, four separate dideoxy "termination" mixtures were prepared in 1.0 ml microcentrifuge tubes. Each mixture (2 µl) contained 20 µM each of the three dNTPs, the remaining dNTP, and its corresponding dideoxy- NTP, being at 2 µM and 200 µM, respectively. Eight microliters of the above labelling reaction mixture was added to each termination mixture and incubated for 15 min at 30°C. Three microliters of stop solution (95% formamide/20 mM EDTA/0.05% xylene cyanol/0.05% bromophenol blue) was then added. The mixtures were heated at 95°C for 2 min immediately prior to loading 6 µl onto a sequencing gel.

A protocol for double-stranded DNA sequence is similar to the above protocol but preceded by an alkali-denaturation step.

For the reasons discussed above, the secondary structure of the DNA template may impede the progress of

DNA polymerases. This may occur either at an pallindromic sequence, which may form a hairpin structure, or at other sequences where an enzyme pauses dependent upon a specific sequence. Because of the high processivity and strand displacement ability of ϕ29 DNA polymerases, the sequencing results obtained with this polymerase are superior to those of the prior art.

In the following methods it is useful to include accessory proteins, such as p5 and p6 in the reaction mix. Preparation of p6 is described by Blanco et al., 62 J. Virol. 4167, 1988. Preparation of p5 was as follows:

E. coli K12ΔH1Δtrp cells carrying the gene 5-containing recombinant plasmid pGM26 or B. subtilis cells infected with the phage ϕ29 mutant sus 14(1242) were used as a source of protein p5 for purification. The protein p5 present in the E. coli extracts amounted to ~1.4% of the total protein after 2.5 h of induction at 42°C, and that present in the B. subtilis extracts was ~2.7% of the total protein.

Ten g of E. coli K12ΔH1Δtrp cells harboring the gene 5-containing recombinant plasmid pGM26, were induced for 2.5 h at 42°C, were ground with alumina (20 g) and extracted with buffer A (50 mM Tris-HCl, pH 7.5 5% glycerol) containing 0.3 M KCl. The lysate was centrifuged for 10 min at 16,500 x g and the pellet reextracted with the same buffer. The two supernatants were pooled and precipitated with ammonium sulfate to 65% saturation. The pellet was dissolved in buffer A, dialyzed against the same buffer, diluted with buffer A + 20% glycerol and passed through a DEAE- cellulose column (2.7 cm x 10 cm) equilibrated with buffer A - 10 mM NaCl. The column was washed first with buffer A + 20% glycerol, then with buffer A, and protein p5 was

finally luted with buffer A + 50 mM NaCl. The fractions containing protein p5 were pooled and precipitated with ammonium sulfat to 65% saturation. The pellet was resuspended in 1.2 ml of buffer A + 1.4 M ammonium sulfate and 50% glycerol. The pellet remaining after centrifugation, containing most of protein p5, was dissolved in buffer A + 50% glycerol. Protein p5 was purified by a similar procedure from B. subtilis cells infected with the ϕ29 delayed lysis mutant sus14(1242). In all purification steps protein p5 was followed by SDS-polyacrylamide gel electrophoresis.

In some preparations, after the last purification step, protein p5 was centrifuged for 24 h at 260,000 x g at 0°C in a 5 ml 15 to 30% (v/v) glycerol gradient in 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl. After centrifugation, 0.2 ml fractions were collected and the presence of protein p5 was determined.

Example 3: Replicative-type Amplification

As described above, long strands of DNA may be synthesized by primer elongation using a ϕ29-type DNA polymerase. This property may be used to amplify DNA without the need for the temperature cycling used in prior polymerase chain reactions. This process makes use of a protein primer rather than an oligonucleotide primer. Generally, the terminal repeat sequences of a ϕ29-type DNA polymerase are covalently bonded by any of a number of standard methods, to each end of the DNA molecule to be amplified. This bonding may be by direct ligation of the sequence to be amplified, or may be by a procedure similar to site-directed mutagenesis, where an oligonucleotide comprising the terminal sequence is constructed to allow recombination of that nucleotide sequence to adjacent DNA which is required to be amplified. In an alterntive method, a restriction

endonuclease may be used to randomly cut genomic DNA and synthetic oligonucleotides, comprising the terminal sequences, placed at these sites. In each case the DNA is amplified by provision of p2 and p3 proteins, along with nucleoside triphosphates. An example of this now follows:

A preparation of ϕ29 DNA protein-p3 isolated by standard procedure is cut with the restriction nuclease ClaI to produce two fragments of length 6147 and 13138 bp. A DNA fragment containing an appropriate multicloning site is then ligated to join the two ClaI fragments. The DNA fragment to be amplified is then ligated to one of the multicloning sites and the resulting DNA is used as a template for DNA synthesis.

The incubation mixture contains, in 25 µl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM spermidine, 20 mM ammonium sulfate, 80 µM each dCTP, dGTP, dTTP and [α-^{32}P]dATP, 80 ng of ϕ29 DNA polymerase, 20 ng of terminal protein p3 purified as described (Prieto et al. 81 Proc. Natl. Acad. Sci. USA, 1639, 1984) from E. coli N99λts cells harboring the gene 3-containing recombinant plasmid pKC30A1 (Garcia et al., 21 Gene 65, 1983), proteins p5 (9 µg) and p6 (2 µg) purified from ϕ29-infected B. subtilis as described above, and the desired amount of template (10 ng-1 µg). After incubation for 60 min. at 30°C, 25 µl containing all the components of the system except the template are added and the mixture is incubated again for 60 min. at 30°C (2nd cycle). The cycles are repeated in the same way several times.

Example 4: Synthesis of Long Strands of DNA

The DNA polymerases of this invention permit ready synthesis of very long DNA molecules useful in a large number of applications, e.g., RFLP analysis, and

DNA probe construction. There follows an example of this methodology.

Single-stranded M13 DNA was hybridized with a 17-mer M13 oligonucleotide primer. The incubation mixture contained, in 10 µl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1mM DTT, 0.5 µg of primed M13 DNA, 80 µM each dCTP, dGTP, dTTP and [α-^{32}P] dATP and ϕ29 DNA polymerase (50 ng). After incubation for 40 min at 30°C the samples were filtered through Sephadex G-50 spin columns in the presence of 0.1% sodium dodecyl sulfate and the Cerenkov radiation of the excluded fraction was counted. To analyze the size of the DNA synthesized, a sample was subjected to electrophoresis in alkaline 0.7% agarose gels along with DNA length markers. The DNA markers were detected with ethidium bromide and the synthesized DNA was detected by autoradiography of the dried gel. In 40 min of incubation at 30°C, DNA longer than 70 Kb was synthesized.

Other embodiments are within the following claims.

Claims

1. In a method for determining the nucleotide base sequence of a DNA molecule, comprising the steps of:

annealing said DNA molecule with a primer molecule able to hybridize to said DNA molecule;

incubating the annealed mixture in a vessel containing four different deoxynucleoside triphosphates, a DNA polymerase, and one or more DNA synthesis terminating agents which terminate DNA synthesis at a specific nucleotide base, wherein each said agent terminates DNA synthesis at a different nucleotide base; and

separating the DNA products of the incubating reaction according to size, whereby at least a part of the nucleotide base sequence of said DNA can be determined,

the improvement wherein said DNA polymerase comprises a ϕ29 type DNA polymerase.

2. The method of claim 1 wherein said ϕ29-type DNA polymerase is that polymerase in cells infected with a ϕ29-type phage.

3. The method of claim 1 wherein said ϕ29-type DNA polymerase is chosen from ϕ29, Cp-1, PRD1, ϕ15, ϕ21, PZE, PZA, Nf, M2Y, B103, SF5, GA-1, Cp-5, Cp-7, PR4, PR5, PR722, and L17.

4. The method of claim 1 wherein said ϕ29-type DNA polymerase is a modified polymerase

having less than t n p rc nt of the xonuclease activity of the naturally-occurring polymerase.

5. The method of claim 4 wherein said polymerase is modified to have less than one percent of the naturally-occurring exonuclease activity.

6. The method of claim 5 wherein said ϕ29-type DNA polymerase has substantially no exonuclease activity.

7. The method of claim 1 wherein said terminating agent is a dideoxynucleoside triphosphate.

8. A kit for DNA sequencing, comprising:

a ϕ29-type DNA polymerase, and a chain terminating agent.

9. A DNA fragment encoding a modified ϕ29-type DNA polymerase, wherein said polymerase comprises sufficient DNA polymerase activity for use in DNA sequencing, and an exonuclease activity which is less than 10% the activity of the corresponding naturally occurring ϕ29-type DNA polymerase.

10. The fragment of claim 9 wherein said DNA fragment encodes a DNA polymerase having substantially no exonuclease activity.

11. The DNA fragment of claim 9 wherein said DNA fragment comprises a DNA sequence encoding a ϕ29 DNA polymerase in which the amino acid moiety at position 12, 14, or 66 of the polymerase is replaced by an alanine moiety.

presence of a particular nucleotide at the 3' end, i.e., to determine the DNA sequence. However, when the 80,000 dalton protein is used, there is little or no detectable 5'-3' exonuclease activity. Use of this modified Taq polymerase allows the distinct and unambiguous electrophoretic separation of DNA fragments and thereby generates reliable sequence data.

Taq DNA polymerase (both modified and unmodified) has been configured into a DNA sequencing system for enzymatic sequence analysis. This sequencing system, known as the TaqTrack™ (Promega corporation) sequencing system, is a modification of that described by Heiner, C., et al. (1988) Applied Biosystems, Inc. DNA Sequencer Model 370 User Bulletin-Taq Polymerase: Increased Enzyme Versatility in DNA Sequencing, and takes advantage of the intrinsic thermostable properties of the polymerase. The system uses either a radiolabeled oligonucleotide or an internally incorporated radiolabeled dNTP as the signal for autoradiographic detection. In the first case, before hybridizing a primer to the template, the sequencing primer is end-labeled using T4 polynucleotide kinase and an appropriate gamma-labeled nucleotide. A single radiolabel in the newly synthesized DNA strand is attached only to the first nucleotide. In the second case, a radiolabeled nucleotide is incorporated within the length of the newly synthesized DNA strand. Reference is made to Example 1 (infra.) and to TaqTrack™ Sequencing Systems Technical Manual, Promega Corporation, printed October 1988, which is incorporated herein by reference, for a detailed disclosure of this system.

In addition to the problems in DNA sequencing that are associated with an exonuclease type activity there are other commonly encountered difficulties. The modification that accounts for the shift in apparent molecular weight may also alter a number of subtle properties of the polymerase with the net result being that the enzyme produces better DNA sequence products. For example, a modification might additionally enhance the processivity

or the rate of polymerization, change the binding affinity of the polymerase for the template or the nucleotide substrate, or slightly alter the thermostable properties of the enzyme. The modification may also improve the error rate of the polymerase. All of these types of modifications, no matter how slight, may cumulatively have a significant effect on DNA sequencing with the modified polymerase. While the exact number and type of modifications are not yet identified, there is an indisputable correlation between the modification of Taq DNA polymerase to the 80,000 dalton protein and the ability to synthesize more DNA fragments with well-defined 5' ends.

The modified Taq DNA polymerase sequencing system may be utilized for a wide variety of single-stranded (ss) and double-stranded (ds) templates such as amplified DNA, large ds DNA templates, such as lambda, GC-rich templates, and long poly (A) tails. The modified Taq DNA polymerase may also be used for thermocycling sequencing methods, DNA amplification, radioactively labeling DNA, nonradioactively labeling DNA, mutagenesis, second strand cDNA synthesis as well as many other applications where a polymerase is required with effectively no 5'-3' exonuclease activity.

The presence of a 5'-3' exonuclease associated with Taq DNA polymerase is most clearly seen in sequencing denatured ds DNA although the effect is also seen when sequencing ss DNA. Reference is made to Figure 2 which illustrates the expected products of both sequencing methods: end label and extension/label, and the expected products when a 5'-3' exonuclease is present. Figure 2 illustrates how a contaminating 5'-3' exonuclease can produce two DNA strands which terminate at different nucleotides and yet have the same length, creating very ambiguous sequence data. In each case involving exonuclease, the first synthesized strand is unaltered and the other two strands are shortened by two nucleotides at the 5' end. The exonuclease-treated DNA strands may have

the same length but terminate at different nucleotides. The end label strands which were treated with exonuclease are no longer radioactive and are therefore undetectable in autoradiographs.

Reference is now made to Figure 3 which illustrates the actual DNA sequence data using the two different polymerases. Notice should be made to the difficulty in reading the sequence using the 85,000 dalton polymerase and the extension label protocol. The usefulness of the modified polymerase is clearly seen when using the extension label procedure.

An alternative DNA sequencing method which can incorporate the use of the modified Taq DNA polymerase of the present invention involves DNA sequencing using alpha-thiodeoxynucleotides for sequence analysis. This protocol is generally described in Labeit, S. (1987), Methods in Enzymology, Vol. 155, p. 166-179) which is incorporated herein by reference. In this process, the modified Taq DNA polymerase is used to incorporate alpha-thiodeoxynucleotides into an elongating DNA strand which is then selectively degraded whereby at least a part of the nucleotide base sequence of the DNA molecule can be determined.

It is also within the scope of the present invention to incorporate the use of the modified Taq DNA polymerase into other DNA sequencing methods.

Thermocycling Reactions

The modified Taq DNA polymerase is stable during temperature thermocycling. Commonly, a thermocycling reaction is repeatedly cycled between up to 3 temperatures to denature, anneal and replicate the DNA while allowing the polymerase to remain catalytically active. A sequencing reaction using modified Taq DNA polymerase can be conveniently repeated by thermocycling. This increases the amount of radiolabeled products and strengthens the autoradiographic signal. Thermocycling is a method use for DNA amplification as described below.

DNA Amplification

A method for amplifying specific DNA sequences, called the polymerase chain reaction, works well with the thermostable modified Taq DNA polymerase. The amplification reaction, preferably a thermocycling reaction, uses two oligonucleotide primers which hybridize to opposite strands and flank the DNA region to be amplified. The annealed primers are aligned preferably with their 3' ends facing each other such that synthesis by modified Taq DNA polymerase extends across the region of the original DNA template between the two primers. Since each primer is complementary to one of the newly synthesized strands, each new strand can participate as a template in subsequent cycles of primer extension and segment amplification. Therefore, each thermocycle of strand denaturation, primer annealing and enzymatic extension doubles the amount of DNA from the previous cycle. Amplification is useful when a greater amount of nucleic acid is required for an analysis. Thus, this method has specific use for the detection of certain diseases. For example, a tiny sample of DNA from a body fluid containing a small quantity of the desired nucleic acid sequence may be amplified, processed according to techniques known to the art and analyzed via the Southern Blotting technique or DNa sequencing. Reference is made to Lo, Y-M.D., et al. (1988), Nucleic Acids Research, Vol. 16, No. 17, p. 8719 for a general discussion of the amplification procedure.

Radioactively Labeling DNA

The present invention also provides a method for labeling a DNA fragment which includes incubating the DNA fragment with modified Taq DNA polymerase and a labeled deoxyribonucleotide, and an appropriate buffer known to the art.

Mutagenesis

Mutagenesis is a method for introducing nucleotide substitutions or mutations within a DNA sequence to alter a gene or gene product. One method, commonly known as in vitro site-directed mutagenesis, involves introducing a mutation by the use of a synthetic oligonucleotide primer containing the desired mutation. The synthetic oligonucleotide is annealed to a template and acts as a primer for synthesis of a new DNA strand containing the mutation. A polymerase such as modified Taq DNA polymerase is required for synthesis of the new strand. Subsequent amplification of the mutation can be accomplished using amplification or polymerase chain reaction (PCR) procedures. Reference is made to Higuchi, R., et al. (1988), Nucleic Acids Research, Vol. 16, No. 15, pp. 7351-7367. Reference is also made to Mole, Sara E., et al. (1989), Nucleic Acids Research, Vol. 17, No. 8, p. 319.

Another possible mutation method is random mutagenesis using modified Taq DNA polymerase. In this method, a sequence can be randomly mutated by misincorporation of nucleotides as the new DNA strand is synthesized.

Second Strand cDNA Synthesis

Modified Taq DNA polymerase may be used for the synthesis of double-stranded cDNA during cDNA cloning. The general method is described in Maniatis, T., et al. (1982) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, using either DNA polymerase for E. coli or avian reverse transcriptase. Maintaining intact 5' ends on both DNA strands is critical in cDNA synthesis since only full length molecules provide all of the inherent sequence information from the original RNA molecule.

Nonisotopic Labeling of DNA

DNA is normally synthesized in vitro from four nucleotides, dATP, dCTP, dGTP, and dTTP. It is possible to modify these bases and use modified Taq DNA polymerase to recognize and incorporate them during DNA replication. This incorporation is the basis of non-isotopic labeling. The modified or 'labeled' bases may be incorporated using several methods involving DNA synthesis, including the polymerase chain reaction, fill-in reactions, or second strand synthesis from a primed template DNA. Reference is made to Maniatis, et al. (supra) and Mole, et al. (supra for a more detailed description of this method.

Kit for DNA Sequencing

The present invention is also directed to a kit for DNA sequencing, comprising the modified Taq DNA polymerase of the present invention and a reagent necessary for sequencing. The reagent may be deoxyribonucleotides, chain terminating agents, DNA sequencing primers, a reaction buffer and mixtures thereof.

The following examples are illustrative of the present invention and are not intended to limit the invention in any way.

EXAMPLES

Example 1

DNA Sequencing Method Utilizing End-Labeled Primer

This invention is directed to dideoxy sequence analysis using modified Taq DNA polymerase with an end-labeled oligonucleotide primer. Reference is made to TaqTrack™ Sequencing Systems Technical Manual, (supra), for a more detailed disclosure of this system.

There are three rapid and simple steps to this approach: 1) the sequencing primer is end-labeled in the presence of T4 polynucleotide kinase and an appropriate gamma-labeled nucleotide; 2) the labeled oligonucleotide and template are hybridized together; and 3) the extension/termination reaction is completed.

The use of an end-labeled primer offers several advantages in sequencing. The single radiolabel per molecule results in a uniform signal intensity throughout the autoradiograph. Internal labeling methods result in increasingly darker signals with increasing length of sequence, which often results in multiple X-ray film exposures and complicates the resolution of closely spaced DNA fragments. Degradation of the sequencing products by radiolysis is not a problem when using end-labeled sequencing primers. When radiolysis occurs, it simply removes the radioactivity from the 5' end of the newly synthesized strand, making it non-radioactive and therefore undetectable in the sequencing autoradiograph. Because of this the end-labeled primers and their extension products may be stored frozen at -20° C for as long as a month and still generate clear sequence data.

Primer Radiolabeling Reaction

Different sequencing strategies require various oligonucleotide primer lengths depending on the temperature of hybridization and complexity of the template. Generally, primers are 15-30 nucleotides long. The protocol is designed to label enough primer for 5 sets of double stranded or 10 sets of single stranded sequencing reactions. The experiment can be scaled proportionately according to the number of reactions to be performed. If the volume of the radiolabel or primer is in excess, it can be concentrated by drying in a vacuum desiccator and redissolving in the appropriate volume of buffer. The amount of radiolabel (^{32}P) in the reaction should be doubled if the isotope has decayed by one-half life (approximately 14 days).

In a microcentrifuge tube, 10 picomole (pmol) primer, 10 pmol gamma-labeled nucleotide, 1 ul T4 polynucleotide kinase buffer, 10X buffer (500 mM Tris-HCl at pH 7.5, 100 mM $MgCl_2$, 50 mM DTT, 1.0 mM spermidine), 0.5 ul T4 polynucleotide kinase (5-10 u/ul) and water to 10 ul is combined. After incubation at 37° C for 10 minutes, the kinase is inactivated at 90° C for 2 minutes and briefly centrifuged. If end-labeling with gamma-^{35}S, 20 units PNK is used while incubating for 4 hours. The end-labeled primers and their extension products may be stored frozen at 20° C for as long as a month and still generate clear sequence data. The primer may be used directly without further purification.

Annealing Plasmid Template and Primer

The recommended amount of ds plasmid template to use per set of sequencing reactions is 1.6 pmol, which represents approximately 4 ug of 3-5 kb plasmid vector. Prior to annealing, the template should be alkali denatured and precipitated. The radiolabeled primer is annealed with the dsDNA plasmid template in an approximately 1:1 molar ratio. For each set of four sequencing reactions, 1.6 pmol of denatured plasmid template, 5.0 ul Taq DNA polymerase 5X buffer (250 mM Tris-HCl, pH 9.0 at 25° C, and 50 mM $MgCl_2$), 2.0 ul labeled primer (2 pmol), and water added to reach a final volume of 25 ul is mixed. The volume is incubated at 37° C for 10 minutes prior to the extension/termination reactions.

Extension/Termination Reactions

For each set of sequencing reactions, four microcentrifuged tubes are labeled with a G, A, T and C. One ul of the corresponding d/ddNTP mix, as described below, is added to each tube and the tubes are capped to prevent evaporation. Six ul of the annealed primer/template mix is added to each of the four tubes containing the d/ddNTP mixes. After all of the tubes are

ready, the Taq DNA polymerase is diluted to 2 u/ul with Taq DNA polymerase buffer which has been diluted to 1X with deionized water. Only enough enzyme required for immediate use should be diluted. For each set of four reactions, 4 ul of diluted enzyme are required. One ul of the diluted enzyme is added to the first tube of labeled primer/template/nucleotide mix. The mixture is mixed by pipetting up and down several times. The mixture is then immediately placed in a temperature of 70° C for 10 minutes. The remaining tubes are prepared accordingly.

When the 10 minutes has expired, the first tube is removed in the series and 4 ul of a stop solution (10 mM NaOH, 95% formamide, 0.05% bromphenol blue, 0.05% xylene cyanole) is added and the tube is set at room temperature. The remaining tubes are prepared accordingly. Before loading a portion of each reaction onto a polyacrylamide sequencing gel, the reactions are heated at 90° C for 5 minutes. Thus, each of the four (A, C, G and T) reaction tubes contains 1 ul d/ddNTP mixture, 6 ul annealed template/primer, 1 ul Taq DNA polymerase (diluted to 2 u/ul) and 4 ul stop solution.

Single-stranded DNA or other denatured ds DNA may be substituted for the plasmid DNA in the above protocol. The recommended amount of ssDNA template to use per set of sequencing reactions is 0.8 pmol or approximately 2 ug of an 8 kb M13 template. The recommended amount of ds lambda phage template to use per set of sequencing reactions is 0.4 pmol, which represents 10 ug of an approximately 42 kb lambda vector. The radiolabeled primer is annealed with the dsDNA lambda template in an approximately 3:1 molar ratio while the annealing of the ssDNA is the same as described above for the denatured plasmid DNA.